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The cardiac atrial appendage stem cell: a new and promising candidate for myocardial repair

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Aims Considerable shortcomings in the treatment of myocardial infarction (MI) still exist and therefore mortality remains high. Cardiac stem cell (CSC) therapy is a promising approach for myocardial repair. However, identification and isolation of candidate CSCs is mainly based on the presence or absence of certain cell surface markers, which suffers from some drawbacks. In order to find a more specific and reliable identification and isolation method, we investigated whether CSCs can be isolated based on the high expression of aldehyde dehydrogenase (ALDH).

Methods and results An ALDH$^+$ stem cell population, the cardiac atrial appendage stem cells (CASCs), was isolated from human atrial appendages. CASCs possess a unique phenotype that is clearly different from c-kit$^+$ CSCs but that seems more related to the recently described cardiac colony-forming-unit fibroblasts. Based on immunophenotype and *in vitro* differentiation studies, we suggest that CASCs are an intrinsic stem cell population and are not mobilized from bone marrow or peripheral blood. Indeed, they possess a clonogenicity of 16% and express pluripotency-associated genes. Furthermore, compared with cardiosphere-derived cells, CASCs possess an enhanced cardiac differentiation capacity. Indeed, differentiated cells express the most important cardiac-specific genes, produce troponin T proteins, and have an electrophysiological behaviour similar to that of adult cardiomyocytes (CMs). Transplanting CASCs in the minipig MI model resulted in extensive cardiomyogenic differentiation without teratoma formation.

Conclusion We have identified a new human CSC population able to differentiate into functional CMs. This opens interesting perspectives for cell therapy in patients with ischaemic heart disease.

Keywords Aldehyde dehydrogenase • Cardiac stem cells • Cardiac differentiation • Myocardial infarction

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1. Introduction

Stem cell therapy is a novel approach to restore cardiac function after myocardial infarction (MI). Over the last decade, the results of clinical trials involving either intracoronary or intramyocardial transplant of bone marrow (BM)-derived stem cells have been published. The minor improvement of cardiac function reported[1,2] may be explained by the limited cardiomyogenic differentiation potential of BM-derived stem cells.[3,4] Nevertheless, transplantation of BM-derived mesenchymal stem cells (MSCs) can be beneficial because it can promote the survival of cardiomyocytes (CMs) through paracrine effects.[5] To develop more successful stem-cell therapies, the scientific focus has shifted from BM-derived stem cells to cardiac stem cells (CSCs) because they are probably programmed to become CMs. The presence of a multipotent endogenous c-kit$^+$ CSC population was first described in rats[6] and later in humans.[7] Since then, several groups have reported the isolation of CSCs expressing a variety of other markers or functional properties, e.g. islet-1,[8] Sca-1,[9] cardiac side population cells,[10] and cardiosphere-derived cells (CDCs).[11] Despite initial promising *in vitro* and pre-clinical research data, the first reported clinical trial with CDCs demonstrated no significant change in cardiac function,[12] making further investigation necessary. To date, two methods have mainly been used to isolate human CSCs: either based on the presence of the c-kit receptor[7] or on

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the formation of cardiospheres and subsequent outgrowth cells, referred to as CDCs.[11] Although isolation of stem cells based on surface antigens has proved to be useful, some limitations arise when cells are isolated based on their phenotypic profile. For instance, the antigen–antibody interaction is susceptible to non-specific binding and strict purity controls are necessary to isolate a pure c-kit$^+$ cell population. More importantly, c-kit is not a unique CSCs marker but is also expressed on hematopoietic stem cells (HSCs) and mast cells, which have been shown to be present in the adult human heart.[13] Furthermore, it cannot be excluded that antibody binding to its receptor influences the cellular properties. Therefore, other research groups have started using CDCs obtained by the functional characteristic of stem cells to generate cardiospheres. However, this isolation method requires a laborious protocol making it rather unattractive for general clinical use. Moreover, sphere formation seems not to be a unique stem-cell characteristic,[14,15] making it unreliable as a stand-alone stem-cell isolation technique. Therefore, the search for new, efficient, and consistent isolation methods is required. A promising strategy for purifying viable stem cells from tissues is based on the aldehyde dehydrogenase (ALDH) enzymatic reaction mechanism. High ALDH activity has already been attributed an important feature of several stem-cell types including mesenchymal, neural and recently also cancer stem cells.[16–18] By using ALDH as an isolation marker, we identified a new ALDH$^+$CD34$^+$CD45$^-$ stem-cell population in human atrial appendages with superior cardiomyogenic differentiation capacity. Because we believe that these cardiac-derived ALDH$^+$CD34$^+$CD45$^-$ cells are probably an intrinsic cell population that is clearly distinct from the previously described c-kit$^+$ CSCs[7], we named these cells cardiac atrial appendage stem cells (CASCs). The identification of this new CASC population in human heart tissue opens interesting perspectives for cell therapy in patients with ischaemic heart disease.

2. Material and methods

All procedures were carried out in accordance with the principles set forth in the Helsinki Declaration. Approval by the institutional review board and informed consent from each patient were obtained. All animal studies were approved by the Hasselt University Institutional Animal Care and Use Committee.

2.1 Isolation and expansion of CASCs from adult human heart tissue

Right atrial appendages were removed during cardiac surgery. The heart tissue was minced, washed, enzymatically dissociated, and stained with Aldefluor® and antibodies according to manufacturer's instructions. ALDH$^+$ cells expressing CD34 but negative for CD45 were directly flow sorted in foetal calf serum (FCS) ($n = 90$). After isolation, cells were seeded in fibronectin-coated culture plates and expanded in X-Vivo 15 medium supplemented with 20% FCS and 2% penicillin–streptomycin (pen-strep).

2.2 Histological analysis of freshly isolated CASCs and c-kit$^+$ CSCs

For cytospins of freshly isolated CASCs and c-kit$^+$ CSCs, a minimum of 1.10^3 cells had to be isolated by flow sorting as previously described. Cytospins were stained with a May-Grünwald Giemsa staining.

2.3 Isolation of CDCs

CDC isolation was performed as previously described but with minor modifications.[11,15]

2.4 Determination of antigen expression profile of non-expanded and *ex vivo* expanded CASCs and CDCs

Single-cell suspensions from heart tissue were incubated with Aldefluor®, and subsequently stained for 30 min in the dark with the antibodies listed in Supplementary material online, *Table S1* ($n = 5$). After *ex vivo* expansion of CASCs and CDCs, cells were trypsinized and 1×10^5 cells/tube were incubated with the same set of antibodies. Flowcytometry was performed on a FACSAria®.

2.5 Flow cytometric analysis of BM and peripheral blood ALDH$^+$ cells

BM was aspirated from patients' sternum before cardiac surgery. The mononuclear cell (MNC) fraction was isolated.[4] The flow cytometric analysis of peripheral blood ALDH$^+$ cells was performed after a red blood cell lysis. For both samples, 5×10^6 cells were dissolved in 1 ml Aldefluor assay buffer, after which they were incubated and analysed ($n = 6$).

2.6 Functional comparison between BM-derived ALDH$^+$ cells and CASCs

ALDH$^+$ cells from BM and heart tissue were stained, isolated, and expanded *ex vivo* as described above. When sufficient cell numbers were obtained, both cell types were forced to differentiate into adipocytes and osteocytes ($n = 3$). HSC cultures were set up by isolating ALDH$^+$ cells directly from BM or heart tissue, followed by immediate seeding in MethoCult® GF H4434.

2.7 Production of GFP containing lentiviruses and infection of cells

Cells were infected with a lentivirus expressing green fluorescent protein (GFP) under the control of a cytomegalovirus promoter (pRRL–CMV–GFP). For the viral production, pRRL–CMV–GFP was co-transfected with pMDLg-RRE, pRSV-REV, and pCMV-VSVG in HEK293-T cells, using EZ lentifect (MellGen Laboratories nv). All plasmids were kindly provided by Dr R.C. Hoeben (University Medical Center, Leiden, The Netherlands).[19]

2.8 Cardiomyogenic differentiation assay

The cardiomyogenic differentiation of CASCs and CDCs was first assessed by mono-culturing these in medium without serum for 1 week ($n = 15$). To further stimulate the cardiomyogenic differentiation, GFP-labelled cells were co-cultured with primary cultures of neonatal rat CMs (NRCMs) under comparable conditions ($n = 20$). For electrophysiology, GFP expressing CASCs were seeded onto contractile CMs and co-cultured for 1–3 weeks prior to the recordings.

2.9 RNA extraction and RT–PCR

Total RNA was isolated using the RNeasy Micro kit (Qiagen). In monocultures, cells were lysed by directly adding RNeasy lysis buffer (RLT) buffer to the tissue culture well after 1 week. In case of co-cultured CASCs, GFP$^+$ cells were purified from the co-culture by flow sorting directly in cooled RLT buffer to maintain optimal RNA quality.

2.10 Immunofluorescence

Paraffin-embedded heart tissue sections were deparaffinized and incubated with a primary mouse–anti-human CD34 (Beckton & Dickinson) and a consecutive goat–anti-mouse FITC (Beckton & Dickinson) antibody. Hereafter, slides were incubated with a rabbit–anti-human cardiac

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troponin T (cTnT) (Abcam), with a subsequent incubation step of sheep–anti-rabbit rhodamine-labelled secondary antibody (Millipore).

For the cell cultures, cells were fixed in 4% paraformaldehyde and permeabilized with 0.3% Triton X (Sigma-Aldrich). Cells were incubated overnight with anti-human cTnT and cardiac troponin I (cTnI) rabbit antibodies (Chemicon) followed by incubation with a sheep–anti-rabbit rhodamine-labelled secondary antibody.

2.11 Electrophysiological recordings

For electrophysiological recordings, mono- and co-cultures were set up as described above. The whole-cell patch clamp technique was used for these experiments,[20] which were performed at room temperature with borosilicate glass patch electrodes.

2.12 *In vivo* differentiation of CASCs in the Göttingen minipig infarct model

All animal experiments conform with the Directive 2010/63/EU of the European Parliament.

For these experiments, six Göttingen minipigs (Ellegaard Göttingen minipigs) were used. CASCs were isolated by flow cytometry from the right atrial appendage of these minipigs removed by right mini-thoracotomy under general anaesthesia. The animals were sedated by an intramuscular injection of ketamine (12.5 mg/kg) and midazolam (0.25 mg/kg). Intravenous access was obtained with a 21- or 22-gauge needle in the ear vein and, initially, anaesthesia was achieved with an intravenous injection of 5 mg propofol. General anaesthesia was maintained with a continuous infusion of propofol 1% at 10 mL/h and remifentanil (100 μg/mL) at a rate of 5 mL/h. Adequacy of anaesthesia was observed by standard anaesthesia monitoring, i.e. pulse oximetry, non-invasive blood pressure, and ECG. The absence of any stress response was considered as adequate anaesthesia.

Porcine CASCs were isolated using the Aldefluor kit® and expanded *ex vivo* under the same conditions as the human-derived CASCs. Before extensive *ex vivo* expansion, porcine CASCs were transfected with a lentiviral construct containing GFP under the control of the eukaryotic promoter pEF1a. After *ex vivo* expansion, GFP^+ CASCs were trypsinized and dissolved in a collagen-matrigelconstruct.[21]

To study the *in vivo* differentiation of the autologously transplanted CASCs, cells were injected intramyocardially after the induction of an MI. Sedation and anaesthesia of the minipigs was identical as described above. The heart was exposed through a sternotomy. The left anterior descending coronary artery was snare occluded for 2 h. Occurrence of a myocardial infarct was visually confirmed with dark discolouration of the cardiac muscle, presence of ST-segment elevation on ECG during occlusion, and increased troponin I levels 6 h after the occlusion. After reperfusion, an average of $18.1 \pm 19.6 \times 10^6$ CASCs were dissolved in 400 μL of collagen-matrigel construct and intramyocardially injected in volumes of 20 μL with a bent 29-gauge needle.

After 2 weeks, the animals were humanely sacrificed. The minipigs were sedated as described above and euthanized by an intravenous overdose of propofol (1%). Hearts were excised and examined 2 weeks after transplantation. They were fixed in 4% formalin and processed for immunofluorescent staining as described above.

2.13 Statistics

Throughout the text, continuous variables are displayed as mean ± SD. For the electrophysiology experiments, the time-dependent currents (I_{to}, $I_{Ca\text{-}L}$, and/or I_{Na}) were measured as the difference between the peak inward and the steady-state current. Values correspond to mean ± SEM and were compared using the Mann–Whitney *U* test.

More detailed protocols can be found in the Supplementary material online.

3. Results

3.1 The human heart contains an $ALDH^+$ stem-cell population

To investigate the presence of an $ALDH^+$ stem-cell population in the adult human heart, single-cell suspensions from right atrial appendages were incubated with Aldefluor® together with antibodies against CD34, CD45, and c-kit. This antibody combination allowed us to discriminate between mobilized HSCs, which are known to be $ALDH^+$,[22] and potential intrinsic cardiac $ALDH^+$ cells. Flow cytometric analysis showed that an average of 0.9 ± 0.8% of the total heart cell population expressed high levels of ALDH (*Figure 1Ab*); of which 83.8 ± 13.4% co-expressed the HSC marker CD34 but lacked the panleukocyte marker CD45 (*Figure 1Ac*).

Unexpectedly, the $ALDH^+CD34^+CD45^-$ population or CASCs turned out to be negative for the previously described CSC marker c-kit (*Figure 1Ca*). To investigate more thoroughly whether $ALDH^+$-$CD34^+CD45^-$ cells form a distinct cell population, a more extensive comparison of the antigen expression profile of both cell populations was performed. In contrast to CASCs, c-kit^+ cells, representing only 0.1 ± 0.1% of the total cell population, displayed a low side scatter (SSC) (*Figure 1Ba*) and were negative for ALDH (*Figure 1Ca*). It should be noted that c-kit^+ cells formed a heterogeneous population that could be divided into two subpopulations. Approximately half of the c-kit^+ cells were negative for CD34 and CD45 (*Figure 1Bb*) corresponding to the phenotype that has been described before by Bearzi *et al.*[7] In addition, these cells turned out to be negative for most markers that were expressed by the CASCs, including CD29, CD55, and CD73 (*Figure 1Cb–d*). A more detailed phenotypic comparison between the freshly isolated CASCs and c-kit^+ CSCs is listed in Supplementary material online, *Table S3*. The other c-kit^+ subpopulation expressed CD45 along with CD34 (*Figure 1Bb*). Hence, these cells were assumed to be cells of hematopoietic origin.

The phenotypic differences between both cell populations were confirmed by a morphological characterization on flow-sorted CASCs and c-kit^+ cells that were stained with May-Grünwald Giemsa (MGG). CASCs represented a homogeneous cell population of MNCs characterized by an irregular shape. The nucleus was round to oval, located peri-centric, and contained some chromatin with vague or clear nucleoli. The cytoplasm was broad, somewhat basophilic without granulation and contained few vacuoles. The nucleus-to-cytoplasm ratio was small (*Figure 1Ad*). In the c-kit^+ fraction, mainly two cell types dominated. On the one hand, large cells with a broad, irregular cytoplasm packed with large granules showing the typical appearance of mast cells were present (*Figure 1Bc*), confirming the previously reported presence of c-kit^+ mast cells in human hearts.[13] On the other hand, smaller cells with round, dark nuclei packed with chromatin structures were detected (*Figure 1Bd*). These cells resembled immature cells and were presumed to be CSCs. *In situ* analysis of CASCs on heart tissue sections indicated that just like the c-kit^+ CSCs, CASCs were not randomly distributed throughout the atrium but could be found in so-called stem-cell niches (*Figure 1D*) as described previously.[7]

Since CASCs represent a new population of CSCs, we investigated whether our cells expressed other cardiac markers like Islet-1[23] and the recently described platelet-derived growth receptor alpha, CD140a.[24] In the $ALDH^+CD34^+CD45^-$ population, an average of 30% of the cells was positive for CD140a while expression of this antigen was not detected in the $ALDH^-$ fraction (see Supplementary

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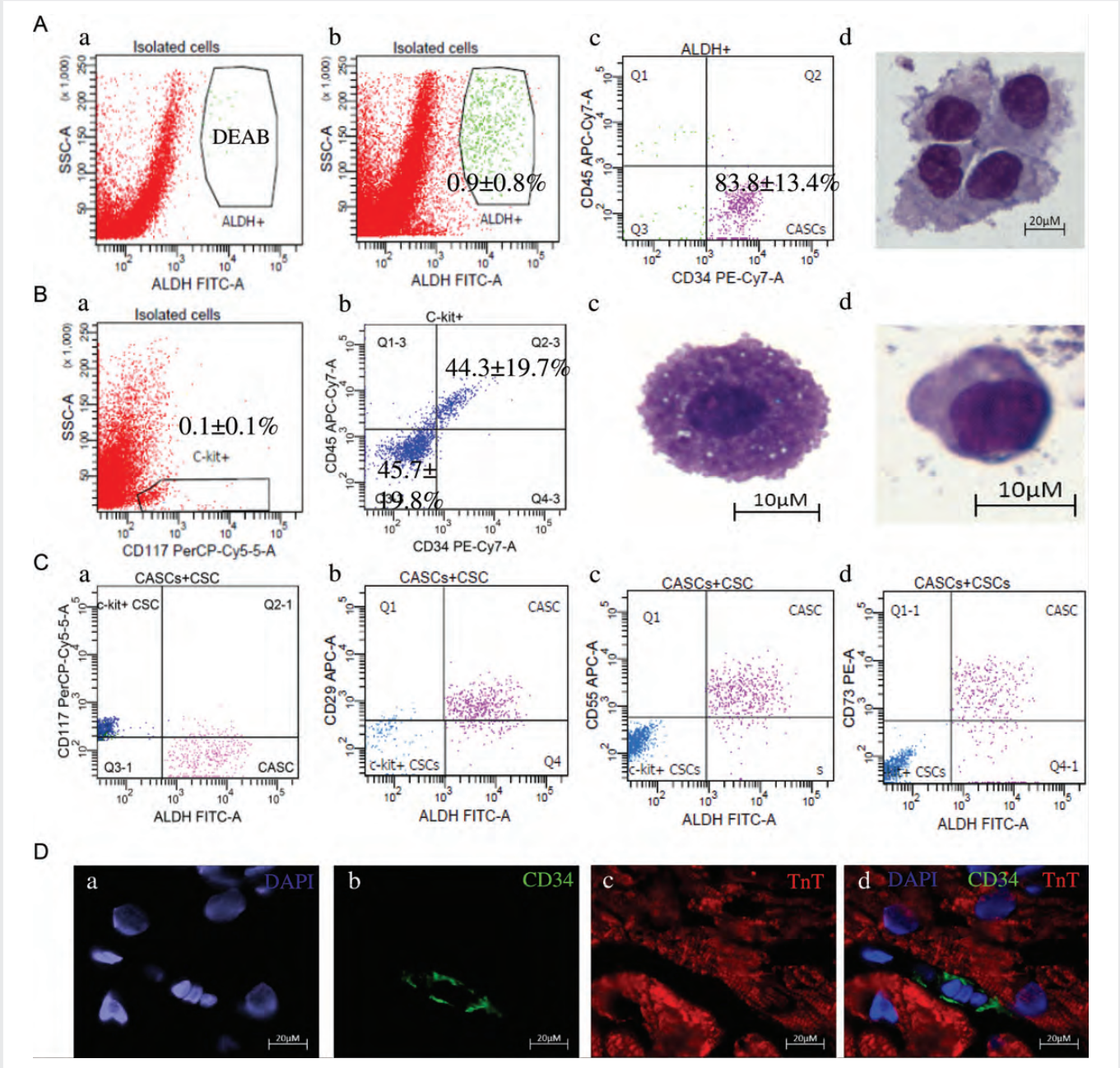

Figure 1 Flow cytometric and morphologic characterization of cells in right atrial heart tissue. (*A*) Analysis of ALDH$^+$ cells. Correct ALDH$^+$ gating was achieved by inhibiting ALDH activity by diethylaminobenzaldehyde (DEAB) (a). Without blocking, 0.9 $\pm$ 0.8% of the total cell population was ALDH$^+$ (b). Of these cells, 83.8 $\pm$ 13.4% expressed CD34 but were CD45$^-$ (c). Freshly isolated CASCs stained by May-Grünwald Giemsa (MGG) showed MNCs with a round to oval nucleus. Their cytoplasm is broad, basophilic, and contains little vacuoles (d). (*B*) Analysis of c-kit$^+$ cells. 0.1 $\pm$ 0.1% of the total cell population expressed CD117 (a). A CD34$^+$/CD45$^+$ and a CD34$^-$/CD45$^-$ subpopulation were identified (b). Flow-sorted c-kit$^+$ cells stained by MGG confirmed the presence of two subpopulations. Large cells with a broad cytoplasm and a small round centrally located nucleus were visible. These cells had the typical appearance of mast cells (c). The second cell population consisted of smaller cells with round, dark nuclei, and displayed a high nucleus/cytoplasm ratio resembling more immature cells (d). (*C*) Phenotypic comparison between ALDH$^+$ cells (pink cell population) and c-kit$^+$ CSCs (blue cell population). In contrast to ALDH$^+$ cells c-kit$^+$ cells were negative for ALDH (a), CD29 (b), CD55 (c), and CD73 (d). (*D*) *In situ* detection of CD34$^+$ cells (green signal; b and d) in atrial heart tissue. Cells are located in so-called stem-cell niches surrounded by adult cardiomyocytes (red signal; c and d) marked by the expression of cardiac troponin T. Nuclei are stained with DAPI (blue; a and d).

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material online, *Figure S1Ac*). Chong *et al.* reported that the colony-forming ability used to examine the clonality of the cells was restricted to the CD140a$^+$ population. However, we could neither detect any difference in number nor in size of the clones between ALDH$^+$-CD140a$^+$ and ALDH$^+$CD140a$^-$ sorted cell fractions (data not shown). In contrast, the other cardiac marker, Islet-1, is uniformly expressed in CASCs and CDCs as shown by immunofluorescence (see Supplementary material online, *Figure S1B*). When flow-sorted CASCs were expanded *ex vivo*, their phenotype changed drastically. The expression of several antigens including CD13, CD44, and CD49c was induced while the expression of CD34 was down-regulated (see Supplementary material online, *Table S4*). As a consequence, *ex vivo* expanded CASCs evolved towards an antigen expression profile that resembled CDCs (see Supplementary material online, *Table S5*) or c-kit$^+$ CSCs after culturing.[7,15,25] Despite the fact that *ex vivo* expanded CASCs lost expression of the CD34 stem-cell marker, Islet-1 and the ALDH activity of these cells persisted at least until cell passage 10 (see Supplementary material online, *Figures S1B* and *S2*).

The elevated ALDH activity in combination with the expression of the stem-cell marker CD34 suggests that CASCs probably possess stem-cell characteristics. To test their clonogenicity, a single-cell sort experiment was performed in which 3456 GFP-labelled CASCs were sorted directly in 96-well plates. Single cell disposition was microscopically analysed and wells with more than one cell were excluded. After 5 days, 582 clones were counted resulting in a clonogenicity of 16.8 $\pm$ 11.4% (*Figure 2A*). In addition, freshly isolated as well as *ex vivo* expanded CASCs expressed pluripotency-associated genes like *Oct-4*, *Nanog*, *c-Myc*, *Klf4 lin-28*, *DPPA3*, and *Tbx3*. However, no expression of *Sox-2* could be detected (*Figure 2B*). Hence, CASCs can be expanded *ex vivo* without losing important stem-cell characteristics.

3.2 CASCs are phenotypically and functionally different from BM stem cells

Since high levels of ALDH expression have been described in stem cells from peripheral blood (PBL) and BM,[16,22] we investigated whether CASCs were not representing mobilized HSCs or MSCs. The antigen expression profile of ALDH$^+$ cells derived from BM or PBL differed noticeably from CASCs. In BM, 85.2 $\pm$ 10.1% of ALDH$^+$ cells (1.2 $\pm$ 0.4%) expressed CD34 and were uniformly positive for CD45 but negative for CD73 (see Supplementary material online, *Figure S3Aa–c*). Similarly, PBL ALDH$^+$ cells (0.8 $\pm$ 0.8%) were all positive for CD45 but negative for CD34 and CD73 (see Supplementary material online, *Figure S3Ad–f*). Moreover, CASCs did not possess the typical functional properties attributed to HSCs or MSCs. For the analysis of the hematopoietic function, freshly isolated BM- and heart tissue-derived ALDH$^+$ cells were seeded in medium containing growth factors stimulating HSC cell growth and differentiation. After 2 weeks, CASCs did not grow in these conditions and remained visible as single cells (see Supplementary material online, *Figure S3Bb*) whereas BM-derived ALDH$^+$ cells formed typical haematological colonies including BFU-E, CFU-GM, and CFU-GEMM (see Supplementary material online, *Figure S3Ba*). To determine mesenchymal characteristics, BM and heart tissue-derived ALDH$^+$ cells were isolated, expanded *ex vivo* under identical culture conditions, and finally forced to differentiate down the adipogenic and osteogenic lineage. In these conditions, BM-derived ALDH$^+$ cells displayed either the typical round morphology of adipocytes containing numerous fat droplets positively stained red with oil-red O (see Supplementary material online, *Figure S3Bc*) or differentiated to osteocytes as shown by a positive alizarin-red staining of Ca^{2+}-deposits (see Supplementary material online, *Figure S3Be*). In contrast, CASCs displayed no signs of adipogenic (see Supplementary material online, *Figure S3Bd*) or osteogenic differentiation (see Supplementary material online, *Figure S3Bf*). Instead, they looked more hypertrophied and demonstrated thick actin bands in the cytoplasm, indicating a more senescent state which was not found in normal *ex vivo* expansion conditions. These results suggest that CASCs are probably not mobilized stem cells from BM.

3.3 CASCs display better cardiomyogenic differentiation than CDCs

To evaluate the differentiation potential of CASCs, their cardiomyogenic differentiation was compared with that of CDCs. For technical and practical reasons, only CDCs were implemented in this comparative assay. Besides, earlier findings showed an equivalent cardiac differentiation potential as c-kit$^+$ CSCs.[15] Initially, cells were monocultured in low serum conditions to stimulate spontaneous cardiomyogenic differentiation. This resulted only in a partial cardiac phenotype shown by the uniform expression of the CM transcription factor *GATA-4*, the structural proteins *Troponin T* (*TnT*) and *α-actinin*, the gap-junctional protein *Cx43* as well as two ion channels, more specifically the potassium voltage-gated channel *Kv4.3* and the L-type calcium channel subunit *α1c*. The expression of *myosin heavy chain* (*MHC*) was not observed (*Figure 3A*). Although monocultured cells expressed *TnT*, the corresponding protein was not translated (data not shown). However, when GFP-labelled cells were brought in a cardiac microenvironment by co-culturing them with neonatal rat CMs (NRCMs), cardiomyogenic differentiation improved but it was much more pronounced in CASCs. In addition to the expression of *TnT*, *α-actinin*, *Cx43*, *Kv4.3*, *α1c*, and *GATA-4*, both cell types also expressed *MHC* after 1 week of co-culturing (*Figure 3A*) as reported earlier for CDCs.[15] However, the superior cardiac differentiation of CASCs compared with CDCs was clearly shown at the protein level by the expression of sarcomeric-organized cTnT (*Figure 3Ba–d*) and cardiac troponin I (cTnI) proteins (*Figure 3C*). In the same experimental setup, CDCs only expressed cTnT (*Figure 3Be–h*) but expression of cTnI could not be observed (data not shown). These observations are consistent with our previously reported data on cardiomyogenic differentiation of CSCs and CDCs.[15] Interestingly, some contracting CASCs could be observed in co-culture while this phenomenon was never noticed with CDCs (see Supplementary material online, *Movie S1*).

As CASCs showed a complete genetic and phenotypic cardiac profile, their electrophysiological properties were investigated by voltage-clamp experiments. *Figure 4a* shows a time-dependent current measured with step depolarizations from a holding potential of -80 mV. Mean data ($n = 5$) of peak and end-of-pulse current (*Figure 4B*) show that time-dependent currents were activated at potentials above -50 mV and suggest the presence of the transient-outward K$^+$ current (I_{to}). This current was recorded in 22% (5 out of 23 cells) of differentiated CASCs. Following a first voltage step to various levels (*Figure 4C* and *D*), the membrane was depolarized in a second step to $+60$ mV to test for the amplitude of the current that was not inactivated during the first step. The measurements were carried out in the absence or presence of 4-aminopyridine (4-AP), which is known to block I_{to}. The time-decaying

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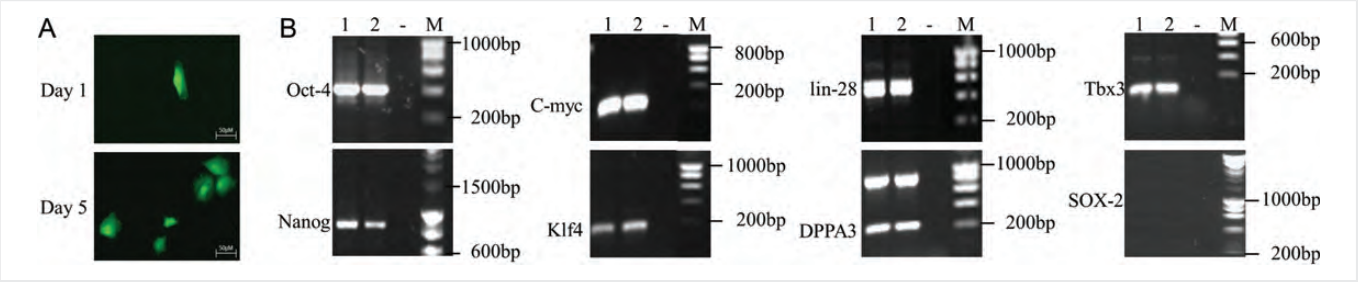

Figure 2 Stem-cell characteristics of freshly isolated and *ex vivo* expanded CASCs. (*A*) Clonogenicity of *ex vivo* expanded CASCs. GFP$^+$ CASCs were sorted with a density of 1 cell/well under stringent purity conditions. Single-cell disposition was confirmed by fluorescent microscopy. After 5 days, 582 clones were counted resulting in a clonogenicity of 16.8 ± 11.4%. (*B*) RT–PCR for the expression of pluripotency associated genes. Lane 1: freshly isolated CASCs; lane 2: *ex vivo* expanded CASCs. (−): negative control; M: marker. CASCs uniformly express *Oct-4*, *NANOG*, *C-myc*, *Klf4*, *lin-28*, *DPPA3*, and *Tbx3*. No expression of *SOX-2* could be detected in any condition.

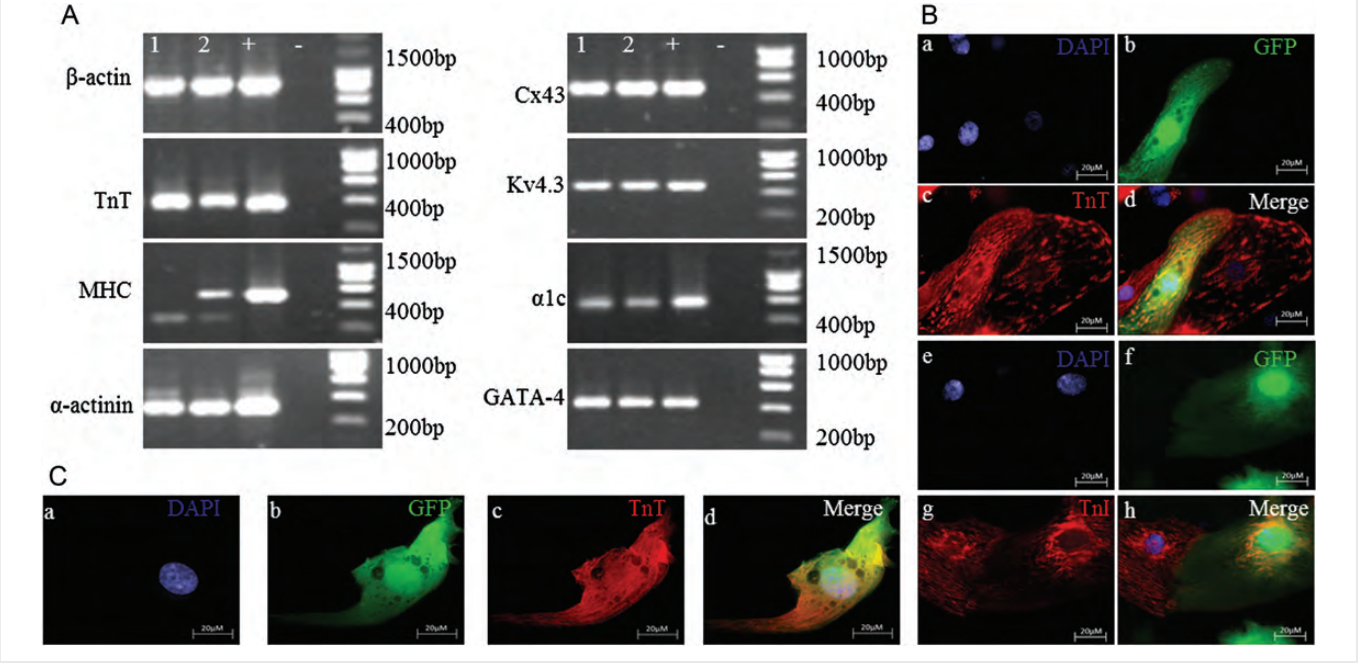

Figure 3 Cardiomyogenic differentiation potential. (*A*) RT–PCR analysis of cardiac specific genes. Lane 1: mono-cultured CASCs; lane 2: co-cultured CASCs; (+): positive control (human cardiomyocytes); −: negative control. Mono- and co-cultured CASCs expressed *GATA-4*, *TnT*, *α-actinin*, *Connexin43 (Cx43)*, *Kv4.3*, and *α1c*. Co-culturing CASCs induced the expression of *MHC*. (*B*) Immunofluorescence of TnT on co-cultured CASCs and CDCs. Co-culturing GFP$^+$ CASCs induced the expression of TnT in CASCs (a–d) and CDCs (e–h) in a sarcomeric organization resembling adult cardiomyocytes. (*C*) Immunofluorescence of TnI on co-cultured CASCs. After 1 week of co-culture with NRCMs GFP$^+$ CASCs (green; b and d) express TnI (red; c and d) in a sarcomeric fashion as expected in adult cardiomyocytes.

component present under control conditions (*Figure 4C*) was suppressed in the presence of 4-AP (*Figure 4D*), whereas the time-dependent current measured at negative potentials (largely representing I_{K1}) remained unaffected. The inactivation curve obtained by plotting the time-dependent current during the second voltage step as a function of the potential during the first step is typical of I_{to} (*Figure 4E*). In some cells, largely inactivating I_{to} by a pre-pulse to −50 mV allowed visualization of an outward current at +50mV, which resulted in a tail current upon repolarization to −10 mV (*Figure 4F*). This result is consistent with the presence of the delayed rectifier K^+ current I_K, but no pharmacological tool was applied to provide a clearer current separation of I_{ks} and I_{kr}. Inward currents activated upon depolarization were detectable in 34.78% of the cells (8 out of 23 cells). The inward currents typically decayed slowly due to inactivation (*Figure 5A*) and the current–voltage relation was bell-shaped with a maximum current occurring at −10 mV (*Figure 5B*). These are typical characteristics of $I_{Ca\text{-}L}$ ($n = 5$). Although no pharmacological test was applied to ascertain the nature of the channel involved, the amplitude of the current increased following the application of isoproterenol (data not shown). In three additional cells, inward current was activated upon depolarization with kinetics that were too fast to be accounted by $I_{Ca\text{-}L}$ (*Figure 5C*). In this case, the voltage of maximum

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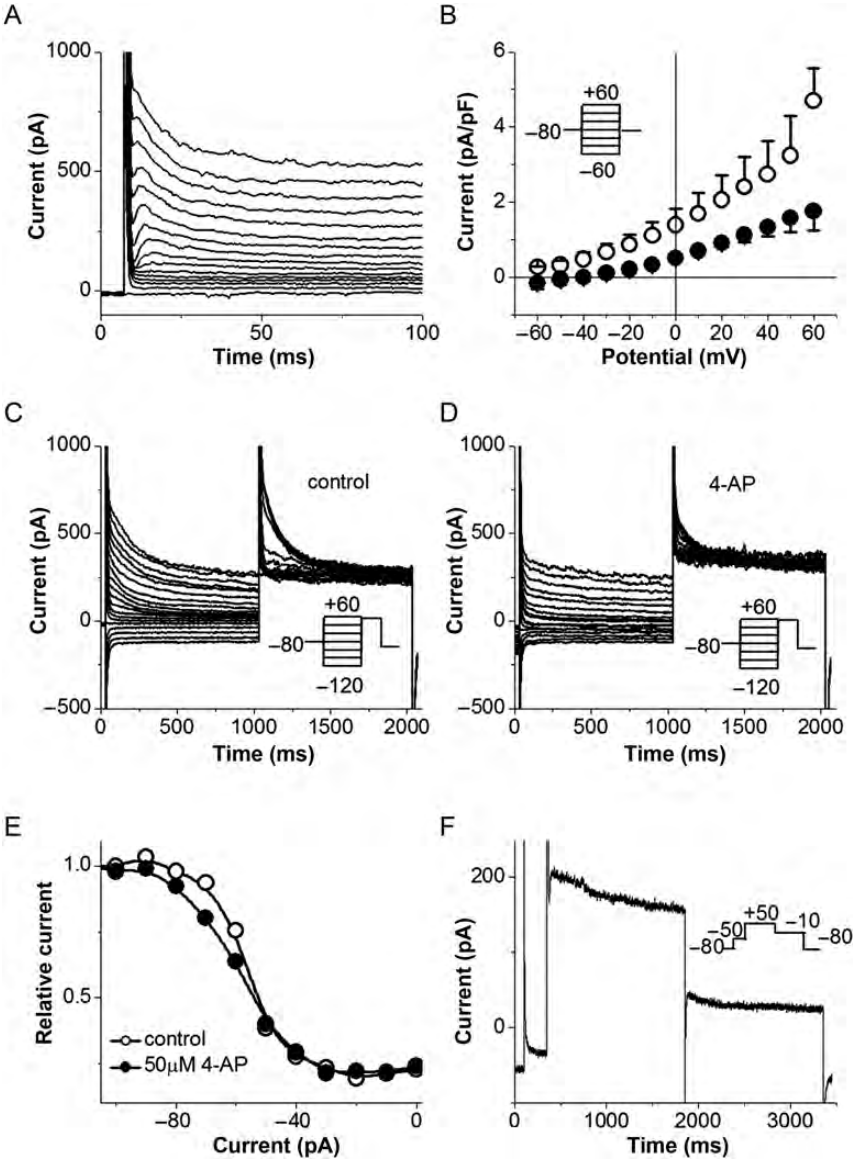

Figure 4 Voltage-activated K^+ currents in co-cultured CASCs. Transient outward current (I_{to}; *A*–*E*). Traces of time-dependent outward currents activated by 100-ms depolarizations from the holding potential (*A*). Means and SEM of currents measured at peak level (unfilled circles) and at the end of the voltage steps (filled circles) (*B*). Currents activated by 1-s depolarizations and tails currents recorded during a second step to +60 mV, in the absence (control) or presence of 50 μM 4-AP, respectively (*C* and *D*). Availability (or inactivation) curves obtained by plotting the relative magnitude of the tails recorded at +60 mV as a function of the potential during the first voltage step. Data from (*C*) and (*D*) (*E*). Depolarization-activated delayed rectifier current (*F*). After largely inactivating I_{to} by a pre-pulse to −50 mV, cells were depolarized to +50 mV to activate both I_{Kr} and I_{Ks} currents followed by a repolarization to −10 mV to deactivate I_{Ks}.

current was also more negative (less than −20 mV; *Figure 5D*), suggesting the presence of either voltage-dependent Na^+ current (I_{Na}) or of the transient Ca^{2+} current ($I_{Ca\text{-}T}$). The presence of voltage-activated inward and outward currents suggested that these cells would be able to display action potentials. All cells had a depolarized resting membrane potential and stimulation under current clamp conditions resulted in a depolarization followed by a slow repolarization (see Supplementary material online, *Figure S4*). Although no maintained plateau was recorded, the response is suggestive of a slow action potential. Besides these currents and slow action potentials showing differentiation of the co-cultured CASCs towards a CM phenotype, the size of differentiated CASCs increased relative to that of the mono-cultured CASCs (see Supplementary material online, *Figure S5*). Cell membrane capacitance of co-cultured CASCs ranged from 64.9 to 314.4 pF, with a mean value of 142.7 ± 16.3 pF ($n = 36$) (see Supplementary material online, *Figure S5*, insert). This was significantly higher than the capacitance of NRCMs (41.3 ± 5.0 pF, $n = 14$; $P < 0.0001$) or mono-cultured CASCs (86.5 ± 10.3 pF, $n = 30$; $P = 0.001$). Thus, capacitance of co-cultured CASCs corresponds to that of mature human cardiac cells.

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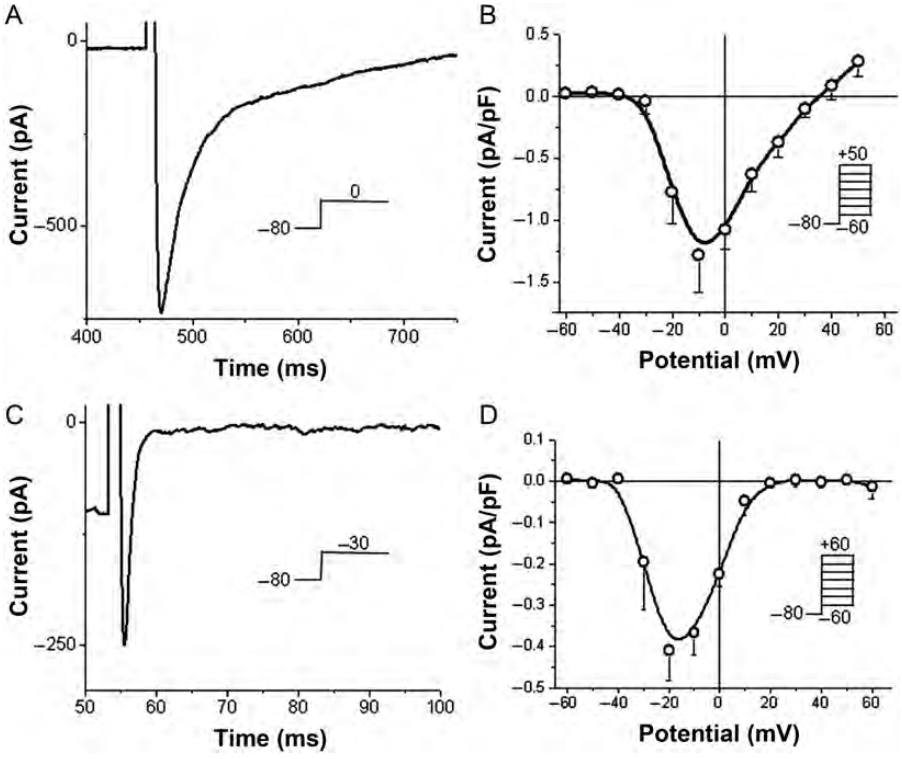

Figure 5 Voltage-activated inward currents in co-cultured CASCs. Trace of current elicited by a 500-ms depolarization from the holding potential of −80 to 0 mV (*A*). Current–voltage relation from experiments such as those of (*A*; $n = 5$), using steps to potentials ranging from −60 to +50 mV in 10-mV steps, suggesting the presence of I_{Ca} (*B*). Trace of fast-decaying inward current elicited by a 100-ms depolarization from the holding potential of −80 to −30 mV (*C*). Current–voltage relation from traces such as those of (*C*; $n = 3$) using steps to various potentials, suggesting the presence of I_{Na} or $I_{Ca\text{-}T}$. In the current–voltage relations (*B* and *D*), current amplitudes were normalized to cell size (current density values, pA/pF) and plotted against test voltages (mV).

3.4 *In vivo* engraftment and differentiation of autologous CASCs

An average of 31.018 ± 15.853 CASCs were isolated from the right atrial appendages of the minipigs (see Supplementary material online, *Figure S6*). After *ex vivo* expansion, an average of 18.1 ± 19.6 × 10^6 autologous cells was intramyocardially transplanted in the peri-infarct zone of an acute MI model.

Immunofluorescence showed successful engraftment after 2 weeks (*Figure 6A* and *B*). Extensive differentiation of the GFP^+ CASCs could be detected by the expression of cTnT (*Figure 6C* and *D*). Importantly, there were no signs of teratoma formation after this follow-up.

4. Discussion

In this paper, we report the existence of a new stem-cell population present in human heart tissue. Since these cells display a unique phenotype, they were named CASCs, after the location where they were first identified. CASCs are mainly characterized by a high ALDH activity, the expression of CD34, and the lack of CD45 and c-kit expression. Despite the previously described potential of CSCs and CDCs to adopt a cardiomyogenic phenotype,[7,11,15] in our hands the isolation and *ex vivo* expansion of CASCs was more reliable and reproducible compared with $c\text{-}kit^+$ CSCs. Moreover, in comparison with CDCs, they showed superior cardiac differentiation capacities.

This is the first time that the presence of an $ALDH^+$ cell population is reported in the human heart. The choice of an enzymatic reaction as an isolation marker for stem cells from human cardiac tissue was actually inevitable as the current isolation methods fall short for reasons discussed in detail in this paper. The major advantages of using an enzymatic reaction for cell detection are that a viable and more homogeneous population can solely be isolated compared with isolations based on the antigen–antibody interaction. Indeed, histological comparison between flow-sorted CASCs and $c\text{-}kit^+$ CSCs demonstrated the heterogenic character of the $c\text{-}kit^+$ population in contrast to the sorted CASCs. This heterogenic cell population and the presence of bound antibody on the cell membrane probably explain the poor efficiency and reproducibility that we encountered during the *ex vivo* expansion of $c\text{-}kit^+$ CSCs.

Meanwhile, it is well known that ALDH activity is a feature shared by several stem-cell types including HSCs and MSCs. Despite the similarity with these cell types, we showed that CASCs constitute an independent cell population with a unique phenotype not displaying the typical HSC and MSC functional characteristics. Despite these convincing results, the origin of CASCs still has to be determined. Indeed, it remains unclear whether these cells are already present in the foetal heart or are mobilized to the heart after birth and have resided there long enough to adopt a more cardiac committed phenotype. The fact

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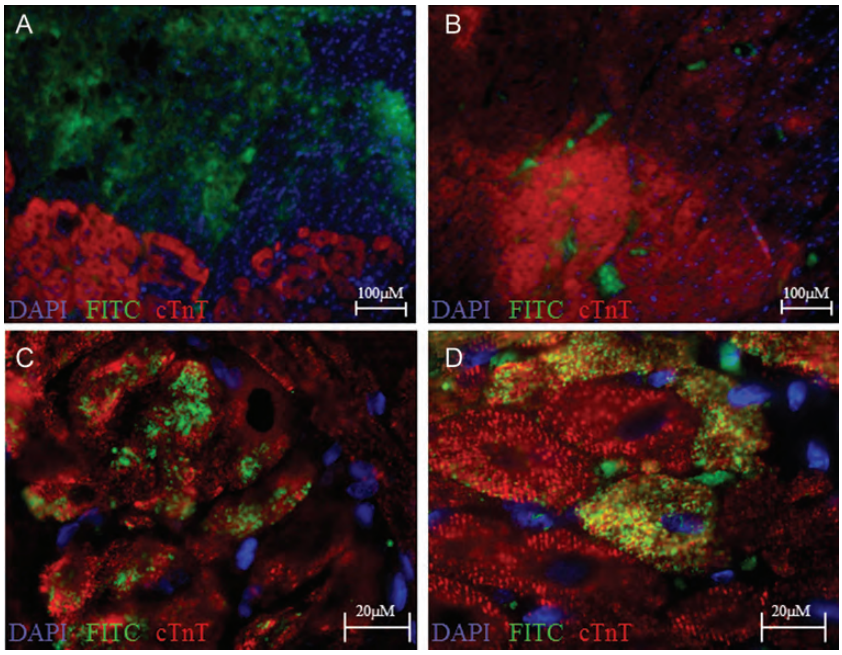

Figure 6 Immunofluorescent analysis on heart slices from CASC transplanted Göttingen minipigs. (*A*) Two weeks after transplantation, large areas of GFP$^+$ CASCs (green) could be detected in the peri-infarct region visible as the border between viable cardiomyocytes, cTnT-positive (red) and cTnT-negative cells, blue nuclei (DAPI). (*B*) Between the viable cardiomyocytes (cTnT$^+$; red), clusters of GFP$^+$ cells (green) are visible. (*C* and *D*) Closer examination of the GFP$^+$ cells (green) demonstrated differentiation towards cardiomyocytes as shown by positive cTnT staining (red).

that CASCs express the typical cardiac marker islet-1 favours the first assumption.

Why high ALDH activity is associated with stem-cell properties is still unknown. *In vivo* ALDH metabolizes aldehydes to their corresponding carboxylic acids[26] and supports the mechanisms of resistance to alkylating agents, e.g. cyclophosphamide.[22,27] Hence a detoxifying role is ascribed to ALDH and cells expressing this enzyme are better protected against certain cytotoxic effects.[22,28] This latter property makes the CASC a perfect candidate for cell therapies in ischaemic-induced diseases. In addition, flow cytometric analysis revealed that these cells were also positive for CD34. Although CD34 was initially used as a HSC-specific marker, expression of this antigen has recently been identified on other tissue-specific cells, e.g. muscle satellite cells[29–31] and, as shown in this paper, also on CASCs. It has become clear that CD34 is important to enhance proliferation and to block differentiation of stem cells.[32] In addition, functions implying chemokine-mediated trafficking and regulation of asymmetric cell division are also ascribed to this marker.[33] Nielsen *et al.* suggested that stem-cell factor and stromal-derived factor, which are both produced as a consequence of myocardial ischaemia, are chemo-attractants for CD34$^+$ cells. We postulate that CASCs may be activated after an ischaemic insult, migrate towards the site of injury and fulfil a protective role. This last assumption is based on the expression of CD73 on CASCs. This 5′-nucleotidase accounts for the rapid conversion of free AMP, which accumulates during ischaemia, into adenosine. It restores the unbalance between the oxygen demand and supply of the heart through its vasodilatory and anti-adrenergic effects.[34] Taken together, all arguments are in favour for the existence of a non-hematopoietic CD34$^+$/CD73$^+$ stem-cell population in the human heart.

Very recently, a new marker for stem cells of cardiac origin has been described: the platelet-derived growth factor receptor also known as CD140a.[24] Since CASCs did not show any similarity with heart stem cells described until now, we wondered whether there was any relationship with this new cCFU-F stem-cell population. Indeed, we found that ~30% of freshly isolated ALDH$^+$CD34$^+$CD45$^-$ cells also expressed this epicardial stem-cell marker while no CD140a$^+$ cells were detected in the ALDH$^-$ fraction. Despite this finding, CFU-F formation was not restricted to the CD140a subfraction since ALDH$^+$CD140a$^-$ cells had an equal colony-forming ability. In the mouse model, expression of CD140a is restricted to stem cells of epicardial origin and only these cells possess a colony-forming ability. Based on our findings, we conclude that ALDH$^+$ heart cells are probably part of the cCFU-F stem-cell hierarchy. According to the hematopoietic model of Christ *et al.*, ALDH activity decreases during HSCs differentiation.[35] If this model can be extrapolated to CSCs, CASCs would be situated in a more immature state compared with CD140$^+$ cells. In support of this theory, CASCs express several pluripotency-associated genes like *Oct-4*, *Nanog*, *c-Myc*, *Klf4*, *lin-28*, *DPPA*, and *Tbx3* which are not detected in cCFU-F stem cells. In addition, the ALDH$^+$/CD140a$^-$ cell fraction possess an equal cCFU-F potential, indicating the presence of multipotent progenitors in this cell fraction.

Since MI results in a massive loss of CMs, replacement by functional tissue is one important aspect of a future treatment. Cardiomyogenic differentiation of *ex vivo* expanded CASCs could only be accomplished by co-culturing these cells with NRCMs, underscoring the importance of a cardiac micro-environment. Early in co-culture, expression of several cardiac-specific genes like *TnT*, *MHC*, *α-actinin* was detected, which was followed by cTnT and cTnI protein

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detection in a sarcomeric organization typical for adult CMs. While co-cultured CASCs displayed almost no ion currents after 1 week, prominent inwardly rectifying and voltage-dependent currents started to develop after 8–10 days. Over time, CASCs differentiated towards an adult CM phenotype as ultimately shown by the presence of I_{Ca}, I_{Na}, I_{to}, I_k, and I_{k1}. Furthermore, the responsiveness to pharmacological drugs like isoprotenerol and 4-AP is of great importance since it demonstrates the presence of functional receptors on differentiated CASCs. The detection of contracting CASCs in co-culture confirmed these results. Cardiac differentiation results of CASCs were far better than those of CDCs since with the latter cells no cTnI could be detected during previous and present *in vitro* differentiation experiments.[15] The limited cardiac differentiation of these CDCs in our experiments could maybe explain the outcome results of the recently published CADUCEUS trial.[12] This proof-of-concept study showed an increase in viable myocardium and, related to this, a decrease in scar size and mass. However, the study failed to detect an increase in left ventricular ejection fraction. Unfortunately, the authors were not able to investigate whether the intracoronary transplanted CDCs homed in the peri-infarcted area of the heart and differentiated to functional adult CMs. The authors claimed that the indirect effect of the transplantation could be more important than the direct differentiation of CDCs to improve cardiac function. Using CASCs for myocardial regeneration may resolve the problem of no direct or complete cell differentiation. Indeed, our *in vivo* experiments demonstrated that after the intramyocardial transplantion of autologous CASCs, these cells were able to survive and engraft in an acute setting of the Göttingen minipig infarct model. Immunofluorescent analysis of tissue slices revealed excellent engraftment of CASCs in the porcine heart 2 weeks after transplantation. A substantial number of the engrafted cells differentiated already towards the cardiomyogenic lineage as shown by a positive staining for cTnT in GFP^+ cells. The survival and differentiation in this very hostile environment of inflammation and fibrosis during the acute phase after the MI proved the potency of the CASCs. However, one should be aware that the engraftment, survival, and differentiation of the CASCs could be positively influenced by the collagen–matrix used for cell injection. Furthermore, despite no signs of teratoma formation could be detected during our observation period, the expression of the pluripotency-associated genes requires a more thorough safety study of the cells, for example in an SCID–mouse model with a longer follow-up. These preliminary data are very promising for potential use of CASCs in a clinical setting. However, a larger preclinical study confirming these results in combination with functional data will be necessary to prove the real potential of the CASCs. In conclusion, we identified a new intrinsic CSCs population that is mainly characterized by high ALDH expression and the presence of CD34 on the plasma membrane. Cells can be expanded *ex vivo* without loss of their pluripotency characteristics and are able to differentiate down the cardiomyogenic lineage *in vitro* as well as *in vivo*. The discovery of this CASC population opens new perspectives for regeneration of the infarcted heart.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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